Biodegradation **10:** 93–104, 1999.

Biodegradation of 2-methyl, 2-ethyl, and 2-hydroxypyridine by an *Arthrobacter* sp. isolated from subsurface sediment

Edward J. O'Loughlin[1,*], Gerald K. Sims[2] & Samuel J. Traina[1]
[1]*School of Natural Resources, Ohio State University, Columbus, Ohio 43210-1086, U.S.A.;* [2]*Present address: USDA/ARS Illinois, University of Illinois, Urbana, Illinois 61801, U.S.A. (*author for correspondence, present address: Air Force Research Laboratory, AFRL/MLQR, 139 Barnes Drive, Suite 2, Tyndall AFB, FL 32403, U.S.A.; e-mail: ed_oloughlin@hotmail.com)*

Accepted 7 December 1998

Key words: 2-ethylpyridine, 2-hydroxypyridine, 2-methylpyridine, biodegradation, *N*-heterocycles

Abstract

A bacterium capable of degrading 2-methylpyridine was isolated by enrichment techniques from subsurface sediments collected from an aquifer located at an industrial site that had been contaminated with pyridine and pyridine derivatives. The isolate, identified as an *Arthrobacter* sp., was capable of utilizing 2-methylpyridine, 2-ethylpyridine, and 2-hydroxypyridine as primary C, N, and energy sources. The isolate was also able to utilize 2-, 3-, and 4-hydroxybenzoate, gentisic acid, protocatechuic acid and catechol, suggesting that it possesses a number of enzymatic pathways for the degradation of aromatic compounds. Degradation of 2-methylpyridine, 2-ethylpyridine, and 2-hydroxypyridine was accompanied by growth of the isolate and release of ammonium into the medium. Degradation of 2-methylpyridine was accompanied by overproduction of riboflavin. A soluble blue pigment was produced by the isolate during the degradation of 2-hydroxypyridine, and may be related to the diazadiphenoquinones reportedly produced by other *Arthrobacter* spp. when grown on 2-hydroxypyridine. When provided with 2-methylpyridine, 2-ethylpyridine, and 2-hydroxypyridine simultaneously, 2-hydroxypyridine was rapidly and preferentially degraded; however there was no apparent biodegradation of either 2-methylpyridine or 2-ethylpyridine until after a seven day lag. The data suggest that there are differences between the pathway for 2-hydroxypyridine degradation and the pathway(s) for 2-methylpyridine and 2-ethylpyridine.

Introduction

Aromatic *N*-heterocycles represent an important class of industrial pollutants, however their environmental fate is not well understood. Though trace levels of these compounds do occur naturally (Sims & O'Loughlin 1989), contamination of surface and groundwaters by pyridine, alkylpyridines, and quinoline has been traced to anthropogenic sources. Aromatic *N*-heterocycles, especially pyridine and alkylpyridines, are often found at locations associated with oil shale (Dobson et al. 1985; Leenheer et al. 1982) and coal processing (Pereira et al. 1983; Stuermer et al. 1982), and at wood treatment sites (Pereira & Rostad 1985); at many such sites, groundwater contamination has been substantial. Of particular concern has been the high water solubility and potential mobility of many *N*-heterocycles through soil (Leenheer & Stuber 1981), which may contribute to groundwater contamination by these compounds.

There have been numerous laboratory studies demonstrating complete mineralization of many aromatic *N*-heterocycles (Sims & O'Loughlin 1989), however many sites show significant contamination by parent compounds (Aislabie et al. 1989; Brockman et al. 1989; Rogers et al. 1985) and hydroxy intermediates (Pereira et al. 1988) several years after the inputs have ceased. Biodegradation of pyridine and quinoline has been observed with pure cultures (Blaschke et al. 1991; Korosteleva et al. 1981; O'Loughlin et al. 1995; Schwarz et al. 1988; Shukla 1986; Shukla & Kaul 1974; Shukla & Kaul 1975; Sims et al. 1986;

Watson & Cain 1975) and native populations in soils and sediments (Bohonos et al. 1977; Godsy et al. 1992; Kuhn & Suflita 1989; Sims & Sommers 1985). By comparison, little is known about the degradation of alkyl substituted pyridines; though at sites containing *N*-heterocycle wastes, they often represent a greater fraction of the contaminant load than either pyridine or quinoline. Complete mineralization of 2-methylpyridine (2-MP) by an *Arthrobacter* sp. (Shukla 1974), 3-methylpyridine by a *Pseudomonas* sp. (Korosteleva et al. 1981), 2-ethylpyridine (2-EP), 3-ethylpyridine, and 4-ethylpyridine by a mixed culture (Feng et al. 1994), and 2-EP, 2,4-dimethylpyridine, and 2,4,6-trimethylpyridine by several *Arthrobacter* spp. (Shukla 1975) has been reported. The purpose of this investigation was to provide insight into the microbial degradation of alkylpyridines. Two-MP was selected for study since it is often the most abundant alkylpyridine in waste streams and at sites contaminated with aromatic *N*-heterocycles (Dobson et al. 1985; Leenheer et al. 1982; Stuermer et al. 1982). The objective of this study was to isolate and characterize a bacterium capable of utilizing 2-MP and to investigate the degradative process.

Materials and methods

Chemicals

Two-MP (98%) and 2-EP (97%) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI); 2-hydroxypyridine (2-HP), riboflavin, and lumichrome were obtained from Sigma Chemical Co. (St. Louis, MO); lumiflavin was obtained from Fluka Chemika-BioChemika (Buchs, Switzerland). Organic solvents were HPLC grade and purchased from J.T. Baker Inc. (Phillipsburg, NJ) and Fisher Scientific (Pittsgurgh, Pa). All other chemicals were reagent grade or better and obtained from Aldrich, Fisher, Fluka, or Sigma.

Media and culture conditions

Unless otherwise indicated, all cultures were maintained in liquid mineral salts medium (MSM) containing 3.35 mmol L^{-1} KCl, 1.04 mmol L^{-1} $MgSO_4$, 2.67 mmol L^{-1} K_2HPO_4, 2.86 mmol L^{-1} KH_2PO_4, and 1 mL L^{-1} trace elements solution (Houghton & Cain 1972), and supplemented with 50 mg L^{-1} Difco® yeast extract (to meet growth factor requirements). The final pH of the MSM was 7.0. Growth substrates were added as indicated. Unless otherwise noted, cultures were maintained at 25–28 °C on a rotary shaker (150 rpm).

Isolation

A bacterium capable of degrading 2-MP was isolated from subsurface sediments collected from an aquifer located at an industrial site in Indianapolis, IN that had been contaminated with pyridine and pyridine derivatives. The sediments, consisting of brown to black, medium to fine sands, were collected at 7–9 m using a split spoon sampler. Five grams of sediment were used to inoculate 200 mL of MSM, containing 5.37 mmol L^{-1} 2-MP as the dominant source of carbon and nitrogen. Following incubation for seven days, 1 mL of the enrichment culture was transferred to fresh medium. On evidence of growth, several 2-MP degrading bacteria were isolated by streak plating on solidified MSM (2% agar) containing 5.37 mmol L^{-1} 2-MP. The isolates were screened for pH tolerance and rate of growth. One of the isolates, designated as R1, was chosen for further study based upon its tolerance of pH values below 7 and its rapid growth on 2-MP. The isolate was maintained as a pure culture on 2-MP agar slants stored under ambient conditions. Slants prepared for long-term storage were maintained at 4 °C and remained viable for > 2 y (without subsequent transfers).

Cell morphology and growth characteristics

Isolate R1 was characterized through a series of morphological, physiological and biochemical tests. Gram reaction and endospore formation were assayed as described by Doetsch (1981). Examination of cell morphology was based upon the method described by Cure & Keddie (1973). Urease activity was assayed by growth response on Christensen urea agar (Smibert & Krieg 1982). Standardized biochemical tests were performed using the API 20E® and 50CHB® diagnostic kits (Analytab Products, Plainview, NY); with *Bacillus polymyxa* used as a control strain. Membrane fatty acid methyl esters were prepared for analysis by the Hewlett-Packard 5898A Microbial Identification System (MIS) according to the procedures outlined by the manufacturer (Hewlett-Packard Corp., Avondale, PA). Quality control guidelines for the operation of MIS specify that cultures should be analyzed 24 h after growth on trypticase soy agar. However, after 24 h isolate R1 exhibited only marginal growth on

this medium. In order to collect enough biomass for sample preparation it was necessary to either collect cells from multiple plates or to extend the incubation times. Both approaches were investigated.

The catabolic activity of the isolate was surveyed over a range of substrates including hydroxy- and alkylpyridines, polycyclic aromatic *N*-heterocycles, substituted benzoates and low molecular weight organic acids. All cultures contained 100 mg L^{-1} of the given substrate (with the exception of acridine, which has an aqueous solubility limit of approximately 32 mg L^{-1}) in MSM containing 0.54 mmol L^{-1} $(NH_4)_2SO_4$, and 30 mg L^{-1} yeast extract, and inoculated with cells grown in MSM containing glucose.

2-Methylpyridine, 2-ethylpyridine, and 2-hydroxypyridine degradation

Growth of the isolate was determined over a range of pH and substrate concentrations. The isolate was cultured in MSM, at pH 4,5,6,7,8, or 9, containing 2.15 mmol L^{-1} 2-MP, 2-EP or 2-hydroxypyridine (2-HP). The isolate was also grown in MSM containing 0, 0.05, 0.10, 1.00, 10.0, or 50.0 mmol L^{-1} 2-MP, 2-EP or 2-HP at pH 7 to determined the dependence of growth on substrate concentration.

Sterile MSM (pH 7.0) containing either 1.20 mmol L^{-1} 2-MP or 2.15 mmol L^{-1} of 2-EP, or 2-HP was inoculated with cells cultured on MSM containing the given substrate which were harvested in log phase by centrifugation. The cells were washed twice, resuspended in MSM, and diluted to a uniform cell density. Media without substrate was inoculated and served as a control for background growth (i.e., growth supported by the yeast extract in MSM). Subsamples were collected at regular intervals and centrifuged; the supernatant was retained for analysis of substrate and ammonium while the pellet was used to measure protein (biomass) as determined by the method of Lowry et al. (1951) standardized with bovine serum albumin. The concentration of 2-MP, 2-EP and 2-HP in the samples was determined by reverse phase HPLC using an Alltech Econosphere® C_{18} 220 by 4.6 mm 5 micron column with UV detection at 262 nm for 2-MP and 2-EP and 226 nm for 2-HP. The mobile phase consisted of 50 : 50, 35 : 65, or 90 : 10 (v : v) 50 mmol L^{-1} ammonium acetate:methanol, for 2-MP, 2-EP and 2-HP respectively, at a flow rate of 1 mL min^{-1}. NH_4^+ was determined by the indophenol blue method (Keeny & Nelson 1982).

Degradation of 2-methylpyridine, 2-ethylpyridine, and 2-hydroxypyridine in a ternary system

Cultures containing MSM amended with 2.15 mmol L^{-1} each of 2-HP, 2-MP, and 2-EP were inoculated with cells grown in MSM containing glucose. Subsamples were collected at regular intervals; biomass and substrate disappearance were determined as previously described.

Characterization of pigment(s) produced during growth on 2-methylpyridine

To assay for production of the yellow pigment observed with growth on 2-MP and 2-EP, 1 L cultures of MSM and MSM containing one of the following at 5.40 mmol L^{-1}, 2-MP, 2-EP, 2-HP, acetate, d-glucose, 4-hydroxybenzoate were prepared. The acetate, glucose, and 4-hydroxybenzoate cultures were amended with 2.74 mmol L^{-1} $(NH_4)_2SO_4$. All cultures were inoculated with cells grown in MSM containing 2.15 mmol L^{-1} 2-MP. When the cultures had reached late log or early stationary phase (3 d), the aqueous phase was recovered by centrifugation, frozen, and lyophilized. The residues were extracted three times with hot (50 °C) methanol and the extracts pooled. One mL each of residue extract, authentic riboflavin in methanol, and authentic lumichrome were spotted on silica gel thin layer chromatography plates (Baker Si250 TLC plates, J.T Baker Inc., Phillipsburg, NJ). The plates were developed and visualized as described by Sims & O'Loughlin (1992).

For spectral analysis, both bacterial pigments and authentic riboflavin and lumichrome were purified by preparative TLC as described above. Fluorescence spectra were obtained with a Perkin Elmer LS-5 scanning fluorescence spectrophotometer. High resolution Fourier transform infrared spectroscopy (FTIR) was performed with a Mattson Polaris instrument (Mattson Instruments, Madison, WI) in the diffuse reflectance mode. Samples for FTIR were prepared with 300 mg IR grade anhydrous KBr.

Results and discussion

Identification and characterization of isolate

The isolate, designated as R1, was gram positive in early growth stages, and became Gram variable in older cultures. When grown in complex media (nutrient agar or glucose/yeast extract) the isolate exhibited

Table 1. Comparison of isolate R1 with related general

	Isolate R1	*Arthrobacter*[1,2]	*Brevibacterium*[1,3]	*Corynebacterium*[1,4]	*Rhodococcus*[2,5]
Rod-coccus growth cycle	+	+	+	+	+
Gram stain	+[6]	+[6]	+	+	+
Motility	+	D[7]	−	−	−
Strictly aerobic	+	+	+	*D*	+
Catalase	+	+	+	+	+
Endospores	−	−	−	−	−
Gelatin hydrolysis	+	+	ND[8]	ND	ND
Starch hydrolysis	−	D	ND	ND	ND
Major fatty acid types	S, A. I[9]	S, A, I	S, A, I	S, U, T	S, U, T
Habitat	Sediment	Soil	Cheese/skin	Human/animal	Soil/dung

[1] Jones & Collins (1986).
[2] Keddie et al. (1986).
[3] Jones & Keddie (1986).
[4] Collins & Cummins (1986).
[5] Goodfellow (1989).
[6] Readily decolorized.
[7] D: Substantial proportion of the species differ.
[8] ND: Not determined.
[9] S: Straight-chain saturated, U: monounsaturated, A: *anteiso*-methyl branched, I: *iso*-methylbranched, T: 10-methyl branched.

a marked rod-coccus growth cycle, however populations in 2-MP media cultures were composed entirely of coccoid cells throughout the growth cycle. The rod forms had a maximum length of 2.5 μm and were generally 0.6 μm in diameter. Coccoid cells were typically 1.2 μm in diameter. Colonies on 2-MP agar were 3–4 mm in diameter, circular, umbonate, smooth, shiny, translucent, and cream in color. The isolate was aerobic, catalase positive, motile, non-spore forming, and did not have a capsule.

The rod-coccus growth cycle exhibited by the isolate is characteristic of a limited number of genera, specifically *Brevibacterium, Arthrobacter, Corynebacterium*, and *Rhodococcus.* A comparison of isolate R1 and these genera is presented in Table 1. Based upon membrane fatty acid types, it was unlikely that isolate R1 belonged in either *Corynebacterium* or *Rhodococcus*. Since R1 was isolated from subsurface sediment (7–9 m below surface) it is unlikely that it is a *Brevibacterium* sp. Isolate R1 appears to be most similar to *Arthrobacter* spp., which are typically isolated from soil.

Attempts to identify the isolate based upon analysis of membrane fatty acid profiles by MIS resulted in multiple matches (MIS System Software 3.2 (Rev 3.2) (Table 2). Matches and their corresponding similarity indices were dependent upon the age of the culture examined. The similarity index is a numerical expression (with a value between 0–1.0) of the correlation between the fatty acid profile of the unknown and the profile of a library match. A similarity index between 0.6 and 1.0 represents an excellent match; that is, there is a high probability that the unknown is related to the library match(es). The isolate was consistently typed as either an *Arthrobacter* sp. or a *Micrococcus* sp. The highest similarity indices were 0.737 for *Micrococcus roseus* at 24 h. and 0.705 for *Arthrobacter globiformus* at 10 d.

Table 2. Characterization of isolate R1 at different culture ages by MIS

		Similarity Index		
Match		24 h[1]	48 h[2]	10 d[2]
Micrococcus		0.564	0.737	0.48
	M. roseus	0.564	0.737	0.48
	M. various	0.356		
Arthrobacter		0.409		0.705
	A. protophormiae	0.409		
	A. globiformus	0.359		0.705
	A. crystallopoietes	0.248		

[1] Results from Clinical Microbiology Laboratory, OSU Hospitals.
[2] Results from the laboratory of Dr. Hoitink, Dept. of Plant Pathology, OARDC Wooster, Ohio.

According to Stackebrandt & Woese (1979) the genus *Micrococcus* is not phylogenetically acceptable. However, based upon the G + C content of the DNA, 16S rRNA sequences, and chemical cell wall analysis, several investigators have suggested that *Micrococcus* is more closely related to *Arthrobacter* than it is to other coccoid genera (Kloos et al. 1974; Kocur et al. 1971; Stackebrandt & Woese 1979). Keddie (1974) has suggested that *Micrococcus* species should be regarded as arthrobacters locked in the coccoid stage of the rod-coccus growth cycle. Since isolate R1 exhibited a rod-coccus growth cycle, it clearly does not belong in the genus *Micrococcus* as it is currently defined. Based upon the results of the morphological, physiological, and chemical tests, isolate R1 has been identified as an *Arthrobacter* species.

Substrate utilization

R1 was able to utilize citrate and simple alcohols and carbohydrates including glycerol, d- and l-arabitol, inositol, mannitol, sorbitol, d- and l-arabinose, ribose, d-xylose, galactose, d-glucose, d-fructose, d-mannose, l-fucose, and trehalose. Starch was not hydrolyzed and there was no indication of urease activity. The catabolic potential of the isolate on a range of substrates is shown in Tables 3–5. With the exception of tartrate, maleamate, and glutamine, isolate R1 exhibited marked growth on the low molecular weight organic acids surveyed, though growth on formate was less substantial. Maleamate is thought to be the focal point for the degradation of many pyridine derivatives including nicotine, nicotinic acid, picolinic acid, and hydroxypyridines (Sims & O'Loughlin 1989). However, the inability of R1 to utilize maleamate does not preclude use of the maleamate pathway for 2-HP degradation by this isolate. The maleamate pathway has been invoked in the degradation of 2-HP by *Arthrobacter* spp. (Shulka 1984), including *A. pyridinolis* and *A. crystallopoietes*, neither of which are able to utilize maleamate as a growth substrate (Ensign & Rittenberg 1963; Kolenbrander et al. 1976). Citrate utilization was also indicated by the API 20E® results. The ability of R1 to utilize 2-, 3-, and 4-hydroxybenzoate, 2,5-dihydroxybenzoate (gentisic acid), 3,4-dihydroxybenzoate (protocatechuic acid) and catechol suggests that it possesses a number of enzymatic pathways for the degradation of aromatic compounds. Marginal growth was observed on 2-hydroxybenzoate and 3,5-dihydroxybenzoate. Phenol, phthalate, and the remainder of the benzoates surveyed restricted growth (relative to the control).

Table 3. Growth of isolate on selected low molecular weight organic acids

Substrate[1]	O.D. 660 nm[2]
Control[3]	0.000 ± 0.005
Formic acid	0.009 ± 0.002
Acetic acid	0.070 ± 0.009
Pyruvic acid	0.052 ± 0.005
Succinic acid	0.049 ± 0.005
Fumaric acid	0.056 ± 0.002
Maleic acid	0.060 ± 0.006
Tartaric acid	-0.011 ± 0.001
Maleamic acid	-0.011 ± 0.001
Propionic acid	0.085 ± 0.004
Citric acid	0.032 ± 0.002
Aspartic acid	0.173 ± 0.011
Glutamic acid	0.153 ± 0.012
Arginine	0.085 ± 0.003
Glutamine	-0.004 ± 0.002

[1] Purity of all chemicals (added at 100 mg L^{-1}) was reagent grade or better.
[2] Mean ± standard deviation (n = 3) relative to control. Values represent maximum measured O.D. normalized to the maximum measured O.D. of the control.
[3] Mineral salts medium containing 30 mg L^{-1} yeast extract.

In contrast to the hydroxybenzoates, R1 was considerably more selective in it's utilization of aromatic *N*-heterocycles; of 32 compounds tested, only 2-MP, 2-EP and 2-HP were degraded. The ability of R1 to degrade substituted pyridine derivatives was highly dependent upon the nature of the functional group and its position on the ring. All of the pyridine derivatives degraded by R1 were substituted in the ortho position. While 2-MP, 2-EP , and 2-HP were readily degraded, 3- and 4-MP, 3-EP, and 3- and 4-HP all inhibited growth. The ability of R1 to utilize alkylpyridines was dependent upon the length of the alkyl chain; optimal growth was observed on 2-MP, 2-EP supported considerably less growth, and 2-propylpyridine inhibited growth. Neither 2-pyridinecarboxaldehyde or 2-pyridinecarboxylic acid were utilized and while 2-HP was degraded, 2-hydroxyquinoline was not. Highly selective substrate utilization is apparently not uncommon; many of the organisms which have been found to degrade specific aromatic *N*-heterocycles

Table 4. Growth of isolate on selected benzoates and hydroxy-aromatic compounds

Substrate[1]	O.D. 660 nm[2]
Control[3]	0.000 ± 0.005
Benzoic acid	0.008 ± 0.001
2-Hydroxybenzoate	0.056 ± 0.0012
3-Hydroxybenzoate	0.125 ± 0.010
4-Hydroxybenzoate	0.131 ± 0.005
2,3-Dihydroxybenzoate	−0.008 ± 0.001
2,4-Dihydroxybenzoate	−0.004± 0.006
2,5-Dihydroxybenzoate	0.082 ± 0.005
2,6-Dihydroxybenzoate	−0.016 ± 0.001
3,4-Dihydroxybenzoate	0.073 ± 0.017
3,5-Dihydroxybenzoate	0.005 ± 0.002
2-Aminobenzoate	−0.015 ± 0.002
4-Aminobenzoate	−0.016 ± 0.003
4-Methoxybenzoate	−0.015 ± 0.001
Phenol	−0.013 ± 0.002
Catechol	0.021 ± 0.003
o-Phthalic acid	−0.018 ± 0.001

[1] Purity of all chemicals (added at 100 mg L^{-1}) was reagent grade or better.
[2] Mean ± standard deviation (n = 3) relative to control. Values represent maximum measured O.D. normalized to the maximum measured O.D. of the control.
[3] Mineral salts medium containing 30 mg L^{-1} yeast extract.

Table 5. Growth of isolate on selected aromatic *N*-heterocycles

Substrate[1]	O.D. 660 nm[2]
Control[3]	0.000 ± 0.005
Pyridine	−0.005 ± 0.004
2-Methylpyridine	0.153 ± 0.001
3-Methylpyridine	−0.010 ± 0.001
4-Methylpyridine	−0.012 ± 0.001
2,3-Dimethylpyridine	−0.012 ± 0.001
2,4-Dimethylpyridine	−0.013 ± 0.002
2,5-Dimethylpyridine	−0.017 ± 0.001
2,6-Dimethylpyridine	−0.012 ± 0.001
3,4-Dimethylpyridine	−0.013 ± 0.001
3,5-Dimethylpyridine	−0.014 ± 0.001
2,4,6-Trimethylpyridine	−0.012 ± 0.001
2-Ethylpyridine	0.063 ± 0.009
3-Ethylpyridine	−0.013 ± 0.002
2-Propylpyridine	−0.011± 0.003
2-Hydroxypyridine	0.092 ± 0.004
3-Hydroxypyridine	−0.021 ± 0.001
4-Hydroxypyridine	−0.020 ± 0.001
2-Pyridinecarboxaldehyde	−0.008 ± 0.001
3-Pyridinecarboxaldehyde	−0.012 ± 0.001
2-Pyridinecarboxylic acid	0.001 ± 0.004
3-Pyridinecarboxylic acid	−0.016 ± 0.004
4-Pyridinecarboxylic acid	−0.010 ± 0.001
3-Hydroxypicolinic acid	−0.008 ± 0.005
3,4-Pyridinedicarboxylic acid	−0.020 ± 0.001
3,5-Pyridinedicarboxylic acid	−0.021 ± 0.001
Quinoline	−0.014 ± 0.002
2-Hydroxyquinoline	−0.034 ± 0.001
4-Hydroxyquinoline	−0.004 ± 0.004
5-Hydroxyquinoline	−0.011 ± 0.001
2,4-Dihydroxyquinoline	−0.018 ± 0.002
Isoquinoline	−0.017 ± 0.001
Acridine	−0.032 ± 0.001

[1] Purity of all chemicals (added at 100 mg L^{-1}) was reagent grade or better.
[2] Mean ± standard deviation (n = 3) relative to control. Values represent maximum measured O.D. normalized to the maximum measured O.D. of the control.
[3] Mineral salts medium containing 30 mg L^{-1} yeast extract.

have shown limited degradative potential for other compounds within this chemical class (O'Loughlin et al. 1995; Shukla 1974; Shukla 1975; Shukla & Kaul 1974; Sims et al. 1986).

2-Methylpyridine degradation

Maximal growth of the isolate on both d-glucose and 2-MP was observed in the range of pH 6–9, peaking at pH 7. However, there were significant differences in growth at pH 5; while there was substantial growth on glucose, growth on 2-MP was almost completely inhibited. Growth in both systems was completely inhibited at pH 4. Since 2-MP is a pH dependent organic cation with a pK_a of 5.92, at pH 5 the protonated species (2-methylpyridinium ion) predominates. The attenuated growth response on 2-MP at pH 5 may have been the result of a preferential uptake of the neutral species or perhaps the lack of an appropriate ion channel or transport system which would allow the 2-methylpyridinium ion to cross the cell membrane.

Isolate R1 was able to utilize 2-MP as the dominant source of C, N, and energy; the yeast extract added to the medium to provide growth factors provided an additional source of carbon, nitrogen, and energy. The growth response of the isolate to 2-MP increased with increasing 2-MP concentration up to 10.7 mmol L^{-1}; above 10.7 mmol L^{-1} growth diminishes with increasing 2-MP concentration. A pale yellow fluorescent pigment was produced during late log to early station-

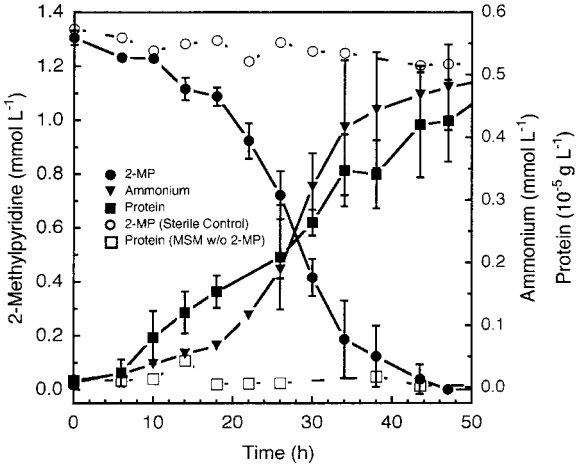

Figure 1. Biodegradation of 2-methylpyridine with the subsequent release of ammonium into the growth medium. Error bars equal one standard deviation.

ary phase. No growth on 2-MP was observed in the absence of molecular oxygen.

The biodegradation of 2-MP by the isolate is presented in Figure 1. Loss of 2-MP was paralleled by growth of the isolate. The concentration of ammonia in the medium was strongly correlated to the biodegradation of 2-MP, suggesting that degradation proceeded at least to the point at which ring nitrogen was released. The non-stoichiometric accumulation of ammonia was attributed to incorporation into biomass and losses from volatilization.

Analysis of the growth medium by reverse phase HPLC provided no evidence of intermediates in the degradative pathway. Although formation of hydroxylated intermediates has been observed during the degradation of quinoline (Bennett et al. 1985; O'Loughlin et al. 1995; Schwarz et al. 1988; Shukla 1986), hydroxy intermediates are not believed to be involved in the degradative pathway of pyridine (Shukla and Kaul 1974; Shukla & Kaul 1975; Sims et al. 1986; Watson & Cain 1975). Cleavage of the pyridine ring is believed to be initiated with partial reduction of the ring. In the tentative pathway proposed by Shukla (1974) for the degradation of 2-MP by an *Arthrobacter* sp., degradation was initiated by the partial reduction of the ring, although no initial metabolites were detected by chromatographic and ultraviolet examination of the culture medium. Degradation terminated with release of CO_2 and NH_4^+, and the incorporation of 2-MP derived carbon and nitrogen into biomass. Though cometabolism of 2-MP by a *Nocardia* sp. (Golovlev 1976) terminated with the oxidation of the methyl group to form picolinic acid (2-pyridinecarboxylic acid), R1 could not use this substrate and there was no evidence of its accumulation in the medium. Isolate R1 was able to utilize 2-hydroxypyridine, catechol, and 2,5- and 3,4-dihydroxybenzoate (gentisic and protocatechuic acid respectively), however, this does not in itself imply that any of these compounds might have a role in the degradative pathway; though it does suggest catabolic diversity. Likewise, the inability of the isolate to utilize picolinic acid does not eliminate the possibility that it is an intermediate in the pathway.

Though no metabolites were detected during 2-MP degradation by R1, the culture medium turned pale yellow in late log phase. Chromatographic analysis of methanol extracts of the lyophilized medium revealed two main chromophores, which on the basis of chromatographic and spectral analysis were identified as riboflavin and lumichrome. Shukla (1974) reported pigment production during 2-MP degradation by an *Arthrobacter sp.* The pigments were identified as riboflavin and a pale yellow compound with a bluish fluorescence in the UV range. It was suggested that the latter compound was a riboflavin degradation product rather than an intermediate in the 2-MP degradative pathway. Though no attempt was made to further identify this compound, its physical properties were similar to lumichrome. The same pigments were produced by this organism during degradation of 2-EP, but not when grown on succinate. Isolate R1 produced riboflavin and lumichrome only when grown on 2-MP. Neither riboflavin nor lumichrome were detected in the methanol extracts of the 2-EP medium residue (or any of the other substrates screened). Overproduction of riboflavin has also been observed during degradation of pyridine by *Micrococcus luteus* (Sims & O'Loughlin 1992). Since riboflavin biosynthesis is thought to be controlled by coordinate repression in bacteria (Brown & Williamson 1982), interference with the regulatory mechanism would likely result in overproduction.

Utilization of 2-Ethylpyridine

Isolate R1 was able to utilize 2-EP as the dominant source of C, N, and energy. The growth response of the isolate to 2-EP increased with increasing 2-EP concentration reaching a maximum at 10.0 mmol L^{-1}. Exposure of the isolate to 2-EP concentration above 50.0 mmol L^{-1} completely inhibited growth. A pale yellow fluorescent pigment was produced during late log to early stationary phase, though it was not as

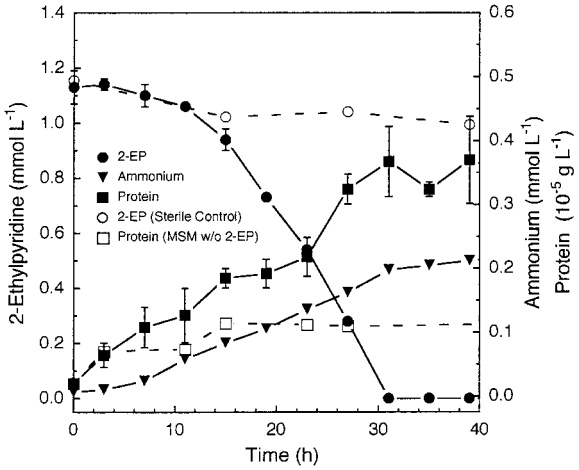

Figure 2. Biodegradation of 2-ethylpyridine with the subsequent release of ammonium into the growth medium. Error bars equal one standard deviation.

intense as in the 2-MP cultures. Neither riboflavin or lumichrome were detected in the medium during growth of R1 on 2-EP. Maximal growth of the isolate on 2-EP was observed in the range of pH 6–9, peaking at pH 7. There were significant differences in the pH-growth response profiles below pH 6 on 2-EP as compared with 2-MP, with significant growth on 2-EP at pH 5. As with 2-MP growth was completely inhibited at pH 4. The degradation of 2-EP by R1 was accompanied by growth and release of NH_4^+ into the medium (Figure 2). The reduced biomass supported by 2-EP relative to 2-MP suggests that 2-EP is a somewhat less suitable substrate for R1. The addition of carbon to the alkyl chain to produce 2-propylpyridine renders the compound toxic to R1; thus it appears that the ability of R1 to utilize ortho-substituted alkylpyridines is highly dependent upon chain length. Though no metabolites were detected by chromatographic analysis, it is clear that 2-EP degradation proceeded to the point at which the ring nitrogen was released and likely progressed to complete mineralization or incorporation into the biomass. Shukla (1974, 1975) has described *Arthrobacter* spp. isolated from soil, which were able to degrade both 2-EP and 2-MP, but was unable to detect any metabolites by chromatographic and spectral analysis. However Feng et al. (1994) have identified the initial step in the degradation of 2-EP by a mixed culture as the oxidation of the aromatic ring resulting in the formation of 2-ethyl-6-hydroxypyridine.

Utilization of 2-Hydroxypyridine

Isolate R1 was able to utilize 2-HP as the dominant source of C, N, and energy. Maximal growth of R1 was observed at 100 mmol L^{-1}. A series of pigments were produced during the course of 2-HP degradation. A bright blue pigment was produced during early log phase. Subsequently the medium changed to purple then to rose and finally to orange, after which there was no apparent change in color. The series of pigments were produced only when 2-HP was present in the media. Color intensity was proportional to the amount of 2-HP added to the media.

Production of pigments during the biodegradation of 2-HP has been reported with other *Arthrobacter* sp. Ensign and Rittenberg (1963) observed the production of a blue pigment during 2-HP degradation by *Arthrobacter crystallopoietes*. In liquid cultures this blue pigment was transitory, and the medium ultimately turned orange-brown in color. A blue pigment with ultraviolet and visible spectra identical to the pigment produced by *A. crystallopoietes* has been reported during degradation of 2-HP by *Arthrobacter pyridinolis* (Kolenbrander et al. 1976). The blue pigment has been identified as 4,5,4′,5′-tetrahydroxy-3′-diazadiphenoquinone-(2,2′) (Kuhn et al. 1965) and is similar to a pigment produced by the spontaneous oxidation of 2,6-dihydroxypyridine (Ensign & Rittenberg 1963). While 2,6-dihydroxypyridine has not been identified as a metabolite in the degradation of 2-HP, it has been identified as an intermediate in the degradation of nicotine by *Arthrobacter oxydans* (Gherna et al. 1965) and during nicotinic acid oxidation by a *Bacillus* sp. (Ensign & Rittenberg 1964). An inducible oxidase which catalyzes the oxidation of 2,6-dihydroxypyridine to 2,3,6-trihydroxypyridine has been identified in *A. oxydans* (Holmes & Rittenberg 1972; Holmes et al. 1972). Concurrent with this reaction is the formation of a blue pigment with spectral properties similar to 4,5,4′,5′-tetrahydroxy-3′-diazadiphenoquinone-(2,2′) which is formed by the spontaneous oxidation of 2,3,6-trihydroxypyridine in air. It is likely that the blue pigment produced by R1 on 2-HP is an identical or related diaza-diphenoquinone.

Maximal growth of the isolate on 2-HP was observed in the range of pH 5–9, peaking at pH 6. There were significant differences in the pH-growth response profiles below pH 6 on 2-HP as compared with 2-MP and 2-EP. Growth at pH 5 was nearly as great as at pH 6–9, however maximal growth did not occur until after a lag of 5 days. At the conclusion of the ex-

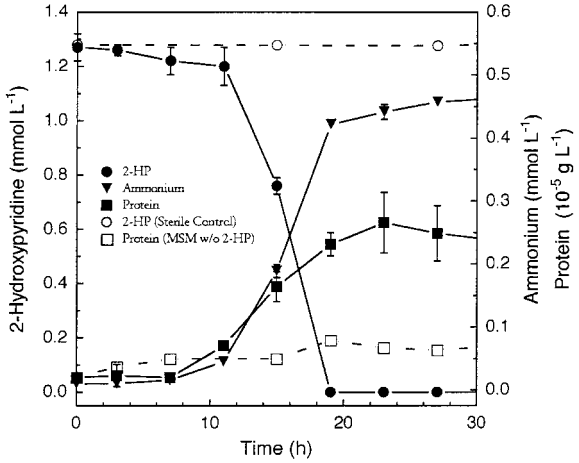

Figure 3. Biodegradation of 2-hydroxypyridine with the subsequent release of ammonium into the growth medium. Error bars equal one standard deviation.

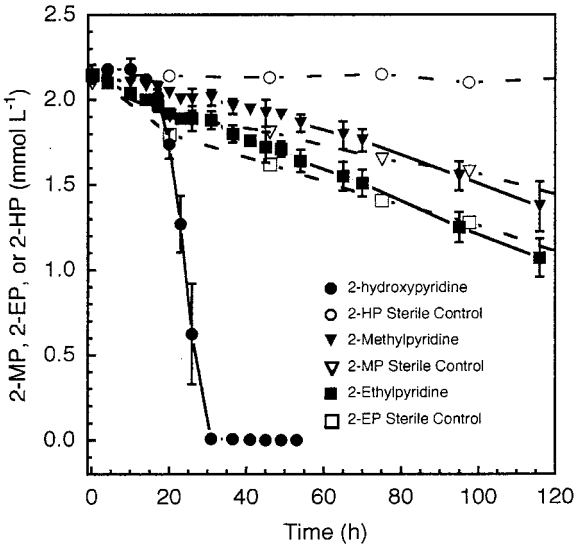

Figure 4. Biodegradation of 2-methyl, 2-ethyl, and 2-hydroxypyridine in a ternary system containing 2.15 mmol L^{-1} of each substrate. Error bars equal one standard deviation.

periment, the cultures with an initial pH of 5 had an average pH of 6.5, suggesting that maximum growth was not achieved until after the pH of the medium had increased closer to 6 (likely the result of release of ammonium into the medium). Pigment production was evident in all pH 5 to 9 cultures with maximum color observed in the pH 6 cultures, however no pigments were observed in the pH 5 cultures until after the 5 day lag. Unlike 2-MP and 2-EP, growth at pH 4 was significant, though greatly diminished relative to the cultures at pH 5 to 9 and there was no evidence of pigment production.

The biodegradation of 2-HP by the isolate is presented in Figure 3. Loss of 2-HP was paralleled by growth of the isolate; however on an equimolar basis, 2-HP supported significantly less biomass than 2-MP and 2-EP. During 2-HP degradation by *A. crystallopoietes* and *A. pyridinolis* pigment production accounts for only approximately 20% and 50% respectively of the 2-HP initially present. Thus it would seem likely that the formation of blue pigment by these organisms and R1 represents a side reaction concurrent with 2-HP mineralization. This is consistent with the decrease in biomass production by R1 on 2-HP relative to 2-MP and 2-EP. As with 2-MP and 2-EP , accumulation of ammonium in the medium was concurrent with 2-HP disappearance, again suggesting that degradation proceeded at least to the point at which ring nitrogen was released. The appearance of blue pigment was first evident 15 hours after inoculation. Analysis of the growth medium by reverse phase HPLC provided no evidence of intermediates in the degradative pathway.

Degradation of 2-MP, 2-EP, and 2-HP in a ternary system

Isolate R1 showed preferential degradation of 2-HP in media containing 2-HP, 2-MP, and 2-EP (Figure 4). After an initial ten hour lag, 2-HP was completely removed within 31 hours of inoculation. Production of the blue pigment was evident at 20 hours after inoculation. There was no apparent biodegradation of either 2-MP or 2-EP through 116 h after inoculation. The loss of 2-MP and 2-EP from the cultures was consistent with their loss through volatilization as observed in the sterile control. At 116 h after inoculation, one of the cultures was reinoculated with 2-MP grown cells. Biodegradation of both 2-MP and 2-EP was evident within 24 h after reinoculation (Figure 5). Biodegradation of 2-MP and 2-EP in the culture which was not reinoculated was not observed until 170 h and 193 h respectively. In both the system which was reinoculated and that which was not, upon initiation of 2-MP and 2-EP degradation, both were rapidly utilized though 2-MP appeared to be degraded more readily than 2-EP. In cultures in which only 2-MP or 2-EP were provided as substrates, degradation was observed within 10 to 20 h after inoculation (Figures 1 and 2). It would appear that some aspect of the 2-HP degradation process temporarily inhibits the induction of the enzyme system(s) involved in 2-MP and 2-EP degradation.

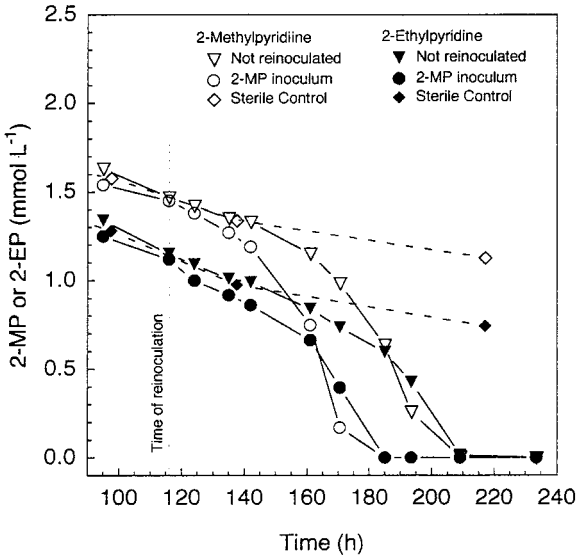

Figure 5. Effect of reinoculation on 2-methyl and 2-ethylpyridine biodegradation in a ternary system.

Conclusions

Isolate R1 appears to be unique among reported 2-MP, 2-EP, and 2-HP degraders, even with respect to the *Arthrobacter* spp. which have been reported. It is the first well-characterized *N*-heterocycle degrading *Arthrobacter* sp. isolated from subsurface material. The 2-MP and 2-EP degrading *Arthrobacter* sp. isolated from soil by Shukla (1974), was unable to utilize 2-HP and did produce riboflavin on both 2-MP and 2-EP (R1 produced riboflavin only on 2-MP). There are also differences between R1 and other reported 2-HP degrading *Arthrobacter* isolates. *A. crystallopoietes* can degrade pyridine but is unable to utilize citrate, while R1 can utilize citrate but is unable to degrade pyridine. *A. pyridinolis* can hydrolyze starch and both *A. crystallopoietes* and *A. pyridinolis* isolates contain the enzyme urease; R1 does not perform either activity.

The data suggest that the pathway for 2-HP degradation in R1 may be separate from the pathway(s) for 2-MP and 2-EP degradation; however, it is possible that most of the pathway is shared and only one enzyme or one regulatory protein is different between the pathways. In addition, the overproduction of riboflavin during 2-MP degradation but not during 2-EP degradation, suggests that there is not complete convergence between the two pathways; however since riboflavin production in bacteria is thought to be controlled by repression, it may be that 2-MP represses riboflavin biosynthesis while 2-EP does not. Further elucidation of the degradative pathway(s) would require studies at the molecular level and is beyond the scope of this investigation.

As compared with other aromatic *N*-heterocycle such as pyridine and quinoline, there have been relatively few well-characterized alkylpyridine degraders described in the literature despite the widespread occurrence of these compounds in the environment; to date, R1 is the only reported well-characterized 2-MP degrader isolated from *N*-heterocycle contaminated material. R1 appears to have an unusually diverse catabolic potential for an *N*-heterocycle degrader. Further study of this organism will be necessary to elucidate the degradative pathways for 2-MP, 2-EP and 2-HP in this organism and to more clearly describe the microbial degradation of alkylpyridines and hydroxypyridines in general.

The isolate has been made available to the research community through the American Type Culture Collection and is designated as *Arthrobacter* sp. ATCC 49987.

Acknowledgments

The authors wish to express their gratitude to Christopher Staron for his assistance in the isolation of R1, and to Michael Boehm and Harold Hoitink of the Dept. of Plant Pathology, OARDC, Wooster, OH and Marilyn Solomon of the Ohio State University Hospitals Clinical Microbiology Unit for their assistance with MIS.

This research was supported in part by a grant from Reily Tar and Chemical Company and by The United States Geological Survey Water Resources Program.

References

Aislabie J, Rothenburger S & Atlas RM (1989) Isolation of microorganisms capable of degrading isoquinoline under earobic conditions. Appl. Environ. Microbiol. 55: 3247–3249.

Bennett JL, Updegraff DM, Pereira WE & Rostad CE (1985) Isolation and identification of four species of quinoline-degrading pseudomonads from a creosote-contaminated site at Pensacola, Florida. Microbios Lett. 29: 147–154.

Blaschke M, Kretzer A, Schäfer C, Nagel M & Andreesen JR (1991) Molybdenum-dependent degradation of quinoline by *Pseudomonas putida* Chin IK and other aerobic bacteria. Arvh. Microbiol. 155: 164–169.

Bohonos J, Chow TW & Spanpggard RJ (1977) Some observations on biodegradation of pollutants in aquatic systems. Jap. J. Antibiot. 30: S275–S285.

Brockman FJ, Denovan BA, Hicks RJ & Fredrickson JK (1989) Isolation and characterization of quinoline-degrading bacteria from subsurface sediments. Appl. Environ. Microbiol. 55: 1029–1032.

Brown GM & Williamson JM (1982) Biosynthesis of riboflavin, folic acid, thiamin, and pantothenic acid. Adv. Enzymol. 53: 345–381.

Collins MD & Cummins CS (1986).Genus *Corynebacterium*. In: P. Sneath (Ed), Bergy's Manual of Determinative Bacteriology, Vol. 2 (pp. 1266–1287). The Williams and Wilkins Co., Baltimore.

Cure GL & Keddie RM (1973) Methods for the morphological examination of aerobic coryneform bacteria. In: Board RG & Lovelock DN (Eds), Sampling – Microbiological Monitoring of Environments (pp. 123–135). Academic Press, New York.

Dobson KR, Stephenson M, Greenfield PF & Bell PRF (1985) Identification and treatability of organics in oil shale retort water. Wat. Res. 19: 849–856.

Doetsch RN (1981) Determinative methods of light microscopy. In: Gerhardt P (Ed), Manual of Methods for General Microbiology (p. 24). American Society for Microbiology, Washington, D.C.

Ensign JC & Rittenberg SC (1963) A crystalline pigment produced from 2-hydroxypyridine by *Arthrobacter crystallopoietes* n. sp. Archiv. Mikrobiol. 47: 137–153.

Ensign JC & Rittenberg SC (1964) The pathway of nicotinic acid oxidation by a *Bacillus* sp. J. Biol. Chem. 239: 2285–2291.

Feng Y, Kaiser J-P, Minard RD & Bollag J-M (1994) Microbial transformation of ethylpyridines. Biodegradation. 5: 121–128. Gherna RL, Richardson SH & Rittenberg SC (1965) J. Biol. Chem. 240: 3669–3674.

Godsy EM, Goerlitz DF & Grbic-Galic D (1992) Methanogenic biodegradation of creosote contaminants in natural and simulated ground-water ecosystems. Ground Water. 30: 232–242.

Golovlev EL (1976) Characteristics of the regulation of the microbial transformation of organic compounds. Chem. Abstr. 85: 156250x.

Goodfellow M (1986) Genus *Rhodococcus*. In: Williams S (Ed), Bergy's Manual of Determinative Bacteriology, Vol. 4 (pp.2362–2371). The Williams and Wilkins Co., Baltimore.

Holmes PE & Rittenberg SC (1972) The bacterial oxidation of nicotine. VII. Partial purification and properties of 2,6-dihydroxypyridine oxidase. J. Biol. Chem. 247: 7622–7627.

Holmes PE, Rittenberg SC & Knackmuss HJ (1972) The bacterial oxidation of nicotine. VIII. Synthesis of 2,3,6-trihydroxypyridine and accumulation and partial characterization of the product of 2,6-dihydroxypyridine oxidation. J. Biol. Chem. 247: 7628–7633.

Houghton C & Cain RB (1972) Formation of pyridinediols (dihydroxypyridines) as intermediates in the degradation of pyridine compounds by micro-organisms. Biochem. J. 130: 879–893.

Jones D & Collins MD (1986) Irregular, nonsporing, Gram-positive rods. In: Sneath P (Ed), Bergy's Manual of Determinative Bacteriology, Vol. 2 (pp. 1261–1266). The Williams and Wilkins Co., Baltimore.

Jones D & Keddie RM (1986) Genus *Brevibacterium*. In: Sneath P (Ed), Bergy's Manual of Determinative Bacteriology, Vol. 2 (pp. 1301–1313). The Williams and Wilkins Co., Baltimore.

Keddie RM (1974) Genus *Arthrobacter*. In: Buchanan and Gibbons (Eds), Bergy's Manual of Determinative Bacteriology (pp. 618–625). 8 edn. The Williams and Wilkins Co., Baltimore.

Keddie RM, Collins MD & Jones D (1986) Genus *Arthrobacter*. In: Sneath, P. (Ed), Bergy's Manual of Determinative Bacteriology, Vol. 2 (pp. 1288–1301). The Williams and Wilkins Co., Baltimore.

Keeny DR & Nelson DW (1982) Nitrogen – Inorganic Forms. In: Page AL (Ed), Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties (pp. 674–676). Soil Science Society of America, Madison

Kloos WE, Tornabene TG & Schleifer KH (1974) Isolation and characterization of micrococci from human skin, including two new species: *Micrococus lylae* and *Micrococcus kristinae*. Int. J. Syst. Bacteriol. 24: 79–101.

Kocur M, Bergan T & Mortensen N (1971) DNA base composition of Gram-positive cocci. J. Gen. Microbiol. 69: 69.

Kolenbrander PE, Lotong N & Ensign JC (1976) Growth and pigment production by *Arthrobacter pyridinolis* n. sp. Arch. Microbiol. 110: 239–245.

Korosteleva LA, Kost AN, Vorob'eva LI, Modyanova LV, Terent'ev PB & Kulikov NS (1981) Microbiological degradation of pyridine and 3-methylpyridine. Appl. Biochem. Microbiol. 17: 276.

Kuhn EP & Suflita JM (1989) Microbial degradation of nitrogen, oxygen and sulfur heterocyclic compounds under anaerobic conditions: Studies with aquifer samples. Environ. Toxicol. Chem. 8: 1149–1158.

Kuhn R, Starr MP, Kuhn DA, Bauer H & Knackmuss HJ (1965) Indigoidine and other bacterial pigments related to 3,3′-bipyridyl. Arch. Mikrobiol. 51: 71–84.

Leenheer JA, Noyes TI & Stuber HA (1982) Determination of polar organic solutes in oil-shale retort water. Environ. Sci. Technol. 16: 714–723.

Leenheer JA & Stuber HA (1981) Migration through soil of organic solutes in an oil-shale process water. Environ. Sci. Technol. 15: 1467–1475.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

O'Loughlin EJ, Kehrmeyer SR & Sims GK (1995) Isolation, characterization and substrate utilization of a quinoline-degrading microorganism. Internat. Biodeterior. Biodegradat. 38: 107–118.

Pereira WE and Rostad CE (1985) Investigations of organic contaminants derived from wood-treatment processes in a sand and gravel aquifer near Pensacola, Florida. U.S. Geological Survey Water-Supply Paper 2290. U.S. Geological Survey, Denver, CO.

Pereira WE, Rostad CE, Garbarino JR & Hult MF (1983) Groundwater contamination by organic bases derived from coal-tar wastes. Environ. Toxicol. Chem. 2: 283–294.

Pereira WE, Rostad CE, Leiker TJ, Updegraff DM & Bennett JL. (1988) Microbial hydroxylation of quinoline in contaminated groundwater: Evidence for incorporation of the oxygen atom of water. Appl. Environ. Microbiol. 54: 827–829.

Rogers JE, Riley RG, Li SW, O'Malley ML & Thomas BL (1985) Microbial transformation of alkylpyridines in groundwater. Wat., Air, Soil Pollut. 24: 443–454.

Schwarz G, Senghas E, Erben A, Schäfer E, Lingens F & Höke H (1988) Microbial metabolism of quinoline and related compounds. I. Isolation and characterization of quinoline-degrading bacteria. System. Appl. Microbiol. 10: 185–190.

Shukla OP (1974) Microbial decomposition of α-picoline. Indian J. Biochem. Biophys. 11: 192–200.

Shukla OP (1975) Microbial decomposition of 2-ethylpyridine, 2,4-lutidine and 2,4,6-collidine. Indian. J. Exp. Biol. 13: 574–575.

Shukla OP (1986) Microbial transformation of quinoline by a *Pseudomonas* sp. Appl. Environ. Microbiol. 51: 1332-1342.

Shukla OP & Kaul SM (1974) A constitutive pyridine degrading system in *Corynebacterium* sp. Indian J. Biochem. Biophys. 11: 201–207.

Shukla OP & Kaul SM (1975) Succinate semialdehyde, an intermediate in the degradation of pyridine by *Brevibacterium* sp. Indian J. Biochem. Biophys. 12: 321–330.

Shulka OP (1984) Microbial transformation of pyridine derivatives. J. Sci. Ind. Res. 43: 98–116.

Sims GK & O'Loughlin EJ (1989) Degradation of pyridines in the environment. Crit. Rev. Environ. Control. 19: 309–340.

Sims GK & O'Loughlin EJ (1992) Riboflavin production during growth of *Micrococcus luteus* on pyridine. Appl. Environ. Microbiol. 58: 3423–3425.

Sims GK & Sommers LE (1985) Degradation of pyridine derivatives in soil. J. Environ. Qual. 14: 580–584.

Sims GK, Sommers LE & Konopka A. (1986). Degradation of pyridine by *Micrococcus luteus* isolated from soil. Appl. Environ. Microbiol. 51: 963–968.

Smibert RM & Krieg NR (1982) General Characterization. In: Gerhardt P (Ed), Manual of Methods for General Microbiology (pp. 433–434). American Society for Microbiology, Washington, D.C.

Stackebrandt E & Woese CR. (1979) A phylogenic dissection of the family *Micrococcaceae*. Curr. Microbiol. 2: 317–322.

Stuermer DH, Ng DJ & Morris CJ (1982) Organic contaminants in groundwater near an underground coal gasification site in northeastern Wyoming. Environ. Sci. Technol. 16: 582–587.

Watson GK & Cain RB (1975) Microbial metabolism of the pyridine ring. Metabolic pathways of pyridine degradation by soil bacteria. Biochem. J. 146: 157–172.